FORM PTO-1390 (REV. 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER: 930008-2008

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO (If known see 37 C.F.R. 1.5): 09/980548

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/03638	20 April 2000 (20.04.00)	23 April 1999 (23.04.99)

TITLE OF INVENTION: **"Promoter-Transactivator System for Inducible High-Level Mammalian Gene Expression With the Option of Cell Growth Control"**

APPLICANT(S) FOR DO/EO/US: **Peter Mueller, Christoph Geserick, Katharina Schroeder, Hansjoerg Hauser**

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 – 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:

declaration (unsigned); Transmittal of Amended Drawings (7 sheets of amended drawings); cover sheet of WIPO Publication; PCT Search Report; PCT Examination Report; PCT Examination Report (corrected version)

EXPRESS MAIL

Mailing Label Number: **EL742671876**

Date of Deposit: **October 19, 2001**

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents and Trademarks, Box PCT Washington, DC 20231.

Ronald R. Santucci
(Typed or printed name of person mailing paper or fee)

(Signature of person mailing paper or fee)

Express Mail No. EL742671876US

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5) 09/980548	INTERNATIONAL APPLICATION NO PCT/EP00/03638	ATTORNEY'S DOCKET NO 930008-2008

21. ☒ The following fees are submitted

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):
Neither international preliminary examination fee (37 CFR 1 482) nor international search fee (37CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00

International preliminary examination fee (37 C F.R 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00

International preliminary examination fee (37 CFR 1 482) not paid to USPTO but international search fee (37 CFR 1 445(a)(2)) paid to USPTO $740 00

International preliminary examination fee paid to USPTO (37 CFR 1 482) but all claims did not satisfy provisions of PCT Article 33(1)-(4 . . . . . $710.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100 00

ENTER APPROPRIATE BASIC FEE AMOUNT = **$ 890.00**

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)). $

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims	4 - 20 =	0	x **$18.00**	$
Independent Claims	1 - 3 =	0	x **$84.00**	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$ 0

TOTAL OF ABOVE CALCULATIONS = $

☐ Applicant claims small entity status. See 37 C.F.R. 1.27. The fees indicated above are reduced by ½. + $

SUBTOTAL = $

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). $

TOTAL NATIONAL FEE = $ 890

Fee for recording the enclosed assignments (37 CFR 1 21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3 28, 3.31). **$40.00** per property + $

TOTAL FEES ENCLOSED = $ 890

Amount to be refunded: $

Charged: $

a. ☒ A check in the amount of $**890** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0320**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. WARNING: Information on this form may become public. **Credit card information should not be included on this form**. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Ronald R. Santucci
Frommer Lawrence & Haug LLP
745 Fifth Avenue
New York, New York 10151

(212)588-0800

SIGNATURE

Ronald R. Santucci
NAME

28,988
REGISTRATION NUMBER

930008-2008

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Peter Mueller, Christoph Geserick, Katharina Schroeder and Hansjoerg Hauser

Serial No.: Not Yet Assigned

International Appln. No.: PCT/EP00/03638

International Filing Date: 20 April 2000 (20.04.00)

Priority Date Claimed: 23 April 1999 (23.04.99)

For: PROMOTER-TRANSACTIVATOR SYSTEM FOR INDUCIBLE HIGH-LEVEL MAMMALIAN GENE EXPRESSION WITH THE OPTION OF CELL GROWTH CONTROL

745 Fifth Avenue
New York, New York 10151

Box PCT
Assistant Commissioner
For Patents
Washington, D.C. 20231

Attn: US/DO/EO

PRELIMINARY AMENDMENT

Dear Sir:

Preliminary to the examination of the above-identified patent application kindly amend the application as follows:

In the Claims:

Kindly rewrite claim 4 as follows:

4. (Amended) Process for inducible high-level mammalian gene expression with the option of cell growth control comprising the steps of

(a) transfecting or transforming mammalian cells with an expression vector or expression vectors, respectively, according to claim 2,

(b) culturing said mammalian cells, or transfected or transformed mammalian cells according to the expression vector(s) of claim 2, in a suitable medium, and,

(c) optionally, controlling the growth of said mammalian cells by varying the concentration and the duration of exposure to estradiol in the medium.

REMARKS

The claims of the above referenced application have been amended to remove all multiple dependencies. No new matter has been added. Accordingly, an early examination of the application is respectfully requested.

The Commissioner is authorized to charge any additional fees that may be required to Deposit Account No. 50-0320.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP

By: [signature]
Ronald R. Santucci
Reg. No. 28,988
(212) 588-0800

APPENDIX:

4. (Amended) Process for inducible high-level mammalian gene expression with the option of cell growth control comprising the steps of

(d) transfecting or transforming mammalian cells with an expression vector or expression vectors, respectively, according to claim 2,

(e) culturing said mammalian cells, or transfected or transformed mammalian cells according to the expression vector(s) of claim 2 [claim 3], in a suitable medium, and,

(f) optionally, controlling the growth of said mammalian cells by varying the concentration and the duration of exposure to estradiol in the medium.

930008-2008

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Peter Mueller, Christoph Geserick, Katharina Schroeder and Hansjoerg Hauser

Serial No.: Not Yet Assigned

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745 Fifth Avenue
New York, New York 10151

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Assistant Commissioner
For Patents
Washington, D.C. 20231

Attn: US/DO/EO

TRANSMITTAL OF AMENDED DRAWINGS

Dear Sir:

Applicant encloses herewith seven (7) drawing sheets (comprising 13 figures). These are the drawings, as amended, during the international phase of the above referenced application.

Respectfully submitted,
FROMMER LAWRENCE &HAUG LLP

By: 
Ronald R. Santucci
Reg. No. 28,988
(212) 588-0800

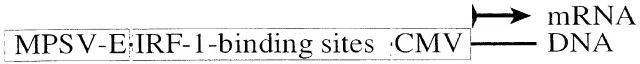


Fig. 1

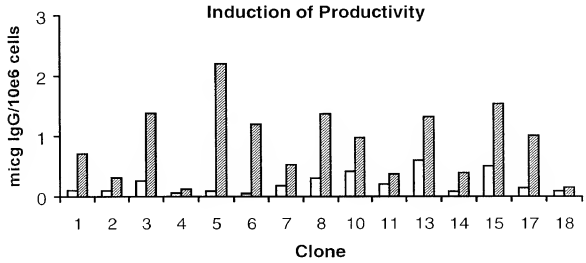


Fig. 2

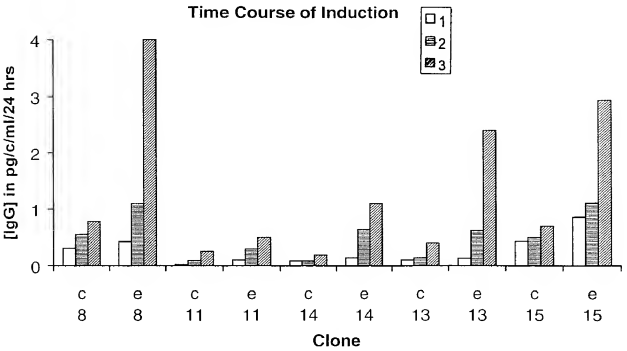


Fig. 3

Fig. 4

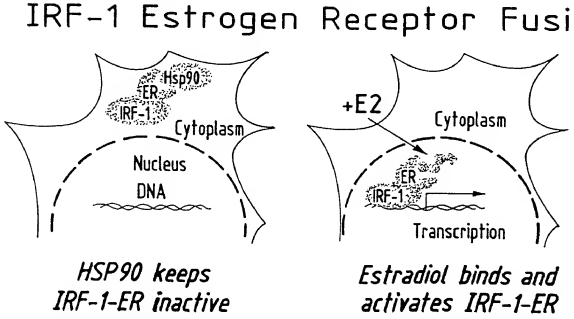


Fig. 5

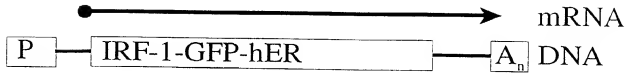


Fig. 6

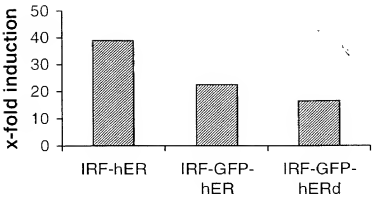


Fig. 7

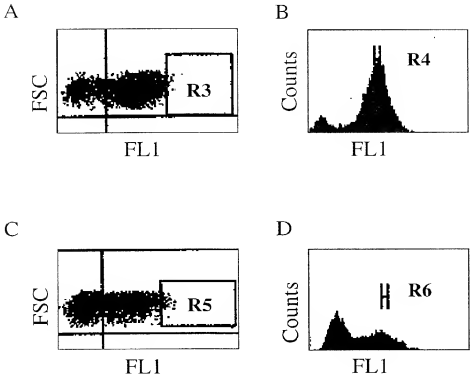


Fig. 8

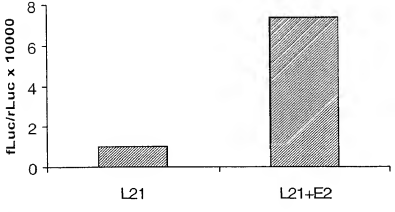


Fig. 9

Fig. 10

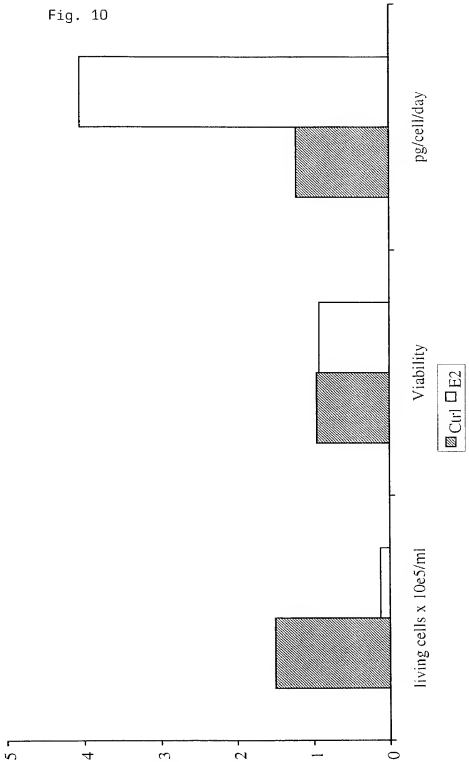


Fig. 11

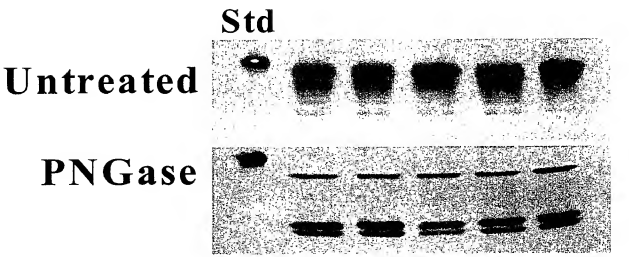
Producer clone Nr. 7
Cells x 10e5/ml; Fraction of viable cells; specific productivity in g/cell/day
0
1
2
3
4
5
living cells x 10e5ml
Viability
pg/cell/day
Ctrl
E2

Fig. 12

Overall Viability
% Viability
100
80
60
40
20
0
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
days of fermentation
Induction with 100 nM E2
overall viability

Product consistency in proliferation-controlled cells

Time [hrs]	24	24	32	24+8	24+24
Estradiol	+	-	-	+/-	+/-



The glycosylation remains constant

Fig. 13

1/PRTS

Promoter-transactivator system for inducible high-level mammalian gene expression with the option of cell growth control

Background of invention

The genetic modification of mammalian cells to express recombinant products is a key strategy in research and biotechnological applications, and mammalian cell cultures have become the preferred production system for secreted pharmaceutical proteins (Hauser, 1997; for complete bibliography of citations see reference list at the end of this specification).

Proliferation control: In the natural environment, cell growth is strictly regulated. Life of a mammal begins with fertilization of the oocyte that divides and proliferates until the animal reaches its mature size. In the adult, proliferation and cell death are balanced to keep the total cell mass essentially constant, while the synthesis and secretion of cellular products continues.

In contrast to the natural situation, due to the requirement to obtain large numbers of cells needed in production processes, mammalian producer cell lines have been selected for rapid and indefinite proliferation capacity. Cell lines used in industrial production processes are transformed and override natural growth control systems such as contact inhibition, anchorage-dependent growth, naturally limited numbers of cell divisions, organ size control and others. Cell growth is a requirement for the genetic manipulation and cloning of cells and then to achieve a sufficient number of producer cells from a single cell initially. However, once a high cell density is reached in a production process, further cell proliferation is associated with disadvantages such as genetic instability, medium depletion, accumulation of toxic products, followed by cell death and cell lysis that leads clogging of cell reten-

tion and product purification devices, product contamination with cellular debris and product deterioration due to glycosidases and proteases (Fussenegger et al., 1999).

Regulated cell growth could mimic the natural situation in technical applications, with rapid proliferation initially until they reach an optimal cell density. Then reduced growth would extend the productive period and keep production conditions constant by lowering medium consumption and waste product accumulation. Since fewer resources are consumed for the synthesis of cell mass, production would be more efficient. The reduced cell division rate would also reduce genetic drift, and by that stabilize the productivity. Therefore, growth regulation could increase production, product quality and consistency.

Genetic growth control systems have specific advantages. There is no need to change production conditions by the addition of toxic compounds, nutrient limitations or suboptimal temperatures that may affect productivity or product quality. The genetic systems are flexible and allow the stepwise improvement of the recombinant regulatory system, optimization of the producer cell and the production conditions. An additional advantage is that the expression of recombinant genes can be induced during growth arrest by using dedicated promoters. Specific requirements must be met by growth control systems concerning stability, productivity, cell viability, quality and consistency of the product and applicability to industrial fermenter systems. Presently under investigation are a system based on cell cycle inhibitory protein overexpression (Fussenegger et al., 1998; Fussenegger et al., 1997; Mazur et al., 1998) and a system using c-jun antisense RNA expression (Kim et al., 1998).

Stabilization of gene expression: The isolation of stable clones is currently a major obstacle for recombinant gene expression in mammalian cells. To date, there is no reliable method that can be afforded by the average research laboratory. In general, in addition to a gene of interest to be

expressed, a marker gene is introduced that facilitates the isolation of producer cell clones. However, not all cells that express the marker gene do also express the gene of interest, and furthermore, the expression-level of the two genes do not necessarily correlate in individual cells, this is a considerable problem especially for the selection of highest-level producer clones and for the selection of cell clones that for an extended time period are to express a protein of interest that has a negative effect on cell growth or viability. The correlation of expression-levels of two genes that are introduced simultaneously depends on the strategy used. The simplest procedure with least tight coupling is the cotransfection method whereby the genes are encoded on different DNA molecules. Tighter coupling is achieved when different genes are coexpressed either from a bi-directional promoter or, preferably, as a polycistronic RNA. Tight coupling of a gene of interest with a selection marker gene ensures stable expression of both genes under selective conditions. This is especially important when the gene of interest imposes a negative selection pressure on the expressing cell, such as proliferation inhibitory genes.

Recombinant gene expression: Often maximizing production is a costly and time-consuming procedure. A most critical factor for productivity is the recombinant gene promoter activity. For constitutive gene expression, a highly active recombinant promoters are available from a variety of sources. These promoters have been derived from cellular and viral genes, from homologous and heterologous systems or they may contain optimized sequences of non-natural origin. For many applications constitutive expression is neither necessary nor desirable. For example in a biotechnological production process, recombinant gene expression in the initial phases of a fermented run may be a metabolic burden on the producer cells, leading to a negative selection pressure of the most productive cells and decrease the performance of the system, while at low cell densities the contribution to the overall production is negligible. Furthermore, product synthesized early may differ in quality from the bulk product synthesized at high cell densi-

ties. Glycoprotein quality depends on the environment of the cells. Since the media compositions, in particular ammonia and glucose concentration, change during the course of fermentations, product glycan structures synthesized early and late in the process differ as well. In addition, secreted products are subject to degradation processes. A long exposure time to degrading enzymes of product synthesized early lead to a lower quality and homogeneity of the product. Therefore, for some applications a system for regulated recombinant gene expression is of advantage. With the exception of the production of highly cytotoxic products, a very high induction rate is not necessary to achieve a higher product quality homogeneity. Despite these advantages, the drawback of regulated gene expression is the generally far less efficient expression compared to the best constitutively active promoters, and a high maximal expression level is of primary interest.

State of the art:

Proliferation control:
To regulate cell growth, IRF-1 (Interferon Regulatory Factor 1) constructs were expressed in mammalian cells. IRF-1 is a transcriptional activator of genes which lead to growth inhibition. To control recombinant IRF-1 activity in vivo, two systems were established: A tetracycline dependent IRF-1 transcription system and a regulatable IRF-1-hER (estrogen receptor fusion protein; Köster et al., 1995; Kirchhoff et al., 1996). The addition of ß-estradiol or other estrogen receptor ligands to the growth medium activates IRF-1-hER, leading to transcriptional activation from promoters containing IRF-1 - binding sites. The expression level depends on the ß-estradiol concentration in the medium and on the duration of the exposure to the hormone (Kirchhoff et al., 1996; Köster et al., 1995). Constitutive expression of IRF-1-hER fusion protein has no effect on cell growth in the absence of the ligand estradiol, and no effect of estradiol on BHK cell growth has been observed in the absence of IRF-1-hER. This proliferation control system has been developed to adjust the cell density of mammalian producer cell cultures (Fussenegger et al., 1999).

Promoters for recombinant gene expression in mammalian cells: Presently two kinds of promoter systems are used for efficient recombinant gene expression. For unregulated expression constitutive promoters are either derived from viral promoters of from strong cellular promoters. For regulated gene expression, tetracycline regulated promoter-transactivator systems have become widely used. This system allows very low basal expression and results in high induced expression levels in many cell lines. However, when compared to strong constitutive promoters, the tetracycline system is not for all cell lines an optimal choice to obtain maximal expression levels.

In one aspect the present invention provides a promoter-transactivator system for inducible high-level mammalian cell gene expression with the option of cell growth control as defined in claim 1.

In a further aspect the present invention provides an expression vector(s) comprising a promoter construct and/or a transactivator construct as defined in claim 1 (i. e. said promotor construct and said transactivator construct may be incorporated in the same expression vector thus containing said a promoter-transactivator system or may be incorporated in separt expression vectors).

In another aspect the present invention provides mammalian cells transfected or transformed with said expression vector(s).

In yet another aspect the present invention provides a process for inducible high-level mammalian gene expression with the option of cell growth control as defined in claim 4.

The invention is now illustrated in more detail.

A system was established that allows regulated high-level gene expression in mammalian cells. It consists of a regulated transcriptional activator IRF-1 and a inducible promoter for high-level recombinant gene expression.

BHK-21 cells were genetically engineered to express regulatable IRF-1 fusion proteins. The inventors investigated several properties that are important to control cell growth in production processes. The regulation was stabilized by using a dicistronic construct to couple the transcription of the IRF-1 gene to an antibiotic resistance selection marker gene. Permanent IRF-1 activation for longer than two to four days lead to viability decreases. To maintain a high cell viability, a schedule was developed of IRF-1 activation and inactivation in several cycles to control cell growth over a period of at least one month while maintaining a high cell viability. Since IRF-1 is a transcriptional activator, promoters containing IRF-1 binding sites can be employed to increase transcription to levels higher than those achieved with strong viral promoters. When IRF-1 is activated, the glycan structure of a relevant pharmaceutical product remained essentially the same and there was no influence on site occupancy or protein integrity.

The present invention is related to:

1) A regulated promoter that shows a higher maximal secreted pharmaceutical protein productivity than a contemporary highly efficient viral promoter (MPSV, myeoloproliferative sarcoma virus). The artificial promoter consists of viral enhancer repeats (MPSV), of IRF-1-binding sites (ISRE-luc, Interferon Stimulated Response Element-firefly luciferase: Kirchhoff and Hauser, 1999) and of a viral minimal promoter element (CMV, cytomegalo virus); (Table 1, Figure 1 and 2).
2) BHK-21 cell lines that expresses an IRF-1-human estrogen receptor (hER) construct whose expression is stabilized by a dicistronic expression construct with a drug resistance gene (puromycin acetyltransferase) as a selection marker (Figure 3).
3) An IRF-1-GFP-hER fusion that can be used for FACS analysis

and sorting of transfected cells (Figure 4). The IRF-hER fusion protein activates promoters with IRF-1-binding sites (for a simplified model see figure 5).

An advantage of the present invention is that secreted recombinant glycoprotein protein quality is maintained in IRF-1 expressing cells (Figure 6).

The promoter (hereinafter also referred to as IRFE promoter) for inducible high-level mammalian gene expression has the composition of Figure 1, wherein

MPSV-E means MPSV enhancer repeats, e. g. from pMBC-1NheI-XhoI, of the sequence:
GCTAGCTTAAGTAACGCCATTTTGCAAGGCATGGGAAAAATACATAACTGAGAATAGAGAAG
TTCAGATCAAGGTCAGGAACAGAGAAACAGGAGAATATGGGCCAAACAGGATATCTGTGGTA
AGCAGTTCCTGCCCCGCTCAGGGCCAAGAACAGTTGGAACAGGAGAATTGGGCCAAACAGGA
TATCTGTGGTAAGCAGTTCCTGCCCCGCTCAGGGCCAAGAACAGATGGTCCCCAGATGCGGT
CCCGCCCTCAGCAGTTTCTAGA,
or isofunctional (i. e. having the same biological activity or function) variants thereof obtained by substitution, insertion or deletion of one or more nucleotides,

IRF-1 binding sites means the sequence:
GATCCCTTCTCGGGAAATGGAAACTGAAAATCAGATCCCTTCTCGGGAAATGGAAACTGAAA
ATCAGATC,
or isofunctional variants thereof obtained by substitution, insertion or deletion of one or more nucleotides,

and **CMV** means the CMV minimal promoter, e. g. from the plasmid pGL2 (Promega, Madison, WI):
TGGCGTGTACGGTGGGAGGCCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAAACCGTCAA
ACCGCGGAAGCT,

or isofunctional variants thereof obtained by substitution, insertion or deletion of one or more nucleotides,

The composite promoter for inducible high-level expression as depicted in the above Figure 1 thus consists of constitutive viral enhancer elements derived from MPSV, IRF-1 binding sequences and a minimal promoter sequence from CMV that determines the RNA start site.

It is appreciated that the above enhancer elements, binding sequences and promoter sequence may be separated by linker or spacer sequences.

Examples and comparative examples

Table 1 shows the basal level expression from the inventive IRFE promoter and a viral promoter

Table 1

Promoter	IgG [mg/ml]
IRFE	1.8 +/- 0.1
IRFE + MPSV	1.7 +/- 0.1
MPSV	1.7 +/- 0.1

From Table 1 it can be seen that the basal level expression from the IRFE promoter is as efficient as a highly active viral promoter. Adherent BHK-21 cells grown in DME medium with 10% FCS were transiently transfected by the calcium cotransfection method with 1 microgram plasmid DNA per ml of culture medium. A sample from the medium supernatant was removed after 24 hrs and the antibody concentration determined by a sandwich ELISA in 96 well microtiter plates. For coating, anti-human IgG (Fab specific) goat antibody (Sigma Immuno Chemicals, No I-5260) was used at a 1:1500-fold dilution. Detection was by a 1:5000 diluted peroxydase conjugated affinity purified goat anti human IgG (H+L) (Jackson ImmunoResearch Laboratories , Dianova, Hamburg, number 109-035-088) followed by a colorimetric peroxydase assay using ortho-phenyldiamine. Human IgG1

Kappa (Sigma, number I-3889) was used as a standard. The absorption at 490 nm was measured in an automatic microplate reader (Cytofluor 2300, Millipore). MPSV: A dicistronic construct with the polio virus IRES element (Sonenberg 1990) expressing recombinant human IgG anti CMV antibody heavy and light chain from the myeloproliferative sarcoma virus promoter (Boldicke et al., 1995; Dirks et al., 1994; Dirks et al., 1993; Arelt et al., 1988). IRFE: the same dicistronic IgG antibody expressed from the IRF-1-inducible promoter. IRFE +MPSV: DNA from both plasmids were mixed at a final concentration of .5 microgram plasmid DNA each per ml of culture medium.

Figure 2 shows the enhanced productivity from the IRFE promoter in IRF-1 induced cells. IRF-1-hER^{3} expressing BHK-21 cells were transfected with DNA encoding an IgG antibody gene that is transcribed from the IRFE promoter. 15 IgG producer clones were identified by a filter immunoassay

(Wirth et al., 1990). Induced IgG expression was determined after 48 hours in the presence of 100 nM estradiol (right bars) or for basal level expression in the absence of estradiol (left bars). IgG concentration in the supernatant was determined by a sandwich ELISA as described in Table 1.

Figure 3 shows that IgG productivity increases gradually after induction. BHK-21 cell clones transfected with IRF-1-hER and IRFE-IgG. IgG expression from an IRF-1 responsive promoter was determined without (c) and with estradiol present in the medium (e) after 1, 2 or 3 days.

Figure 4 shows a IRF-1-hER expression construct . IRF-1-hER is stably expressed from a constitutive viral promoter (P; Dirks et al., 1994) on a dicistronic mRNA

together with puromycin transacetylase as a selection marker gene (PAC; Vara et al., 1986) that is translated due to the presence of a polioviral internal ribosomal entry site (IRES; Sonenberg 1990). The SV40 poly A addition site determines the end of the mRNA. This configuration allows to select transfected cells that efficiently express this construct.

Figure 5 shows the IRF-hER (IRF-ER) protein to be a transcriptional activator that is regulated by ligands such as estradiol (E2; right). It binds to DNA sequences that contain IRF-binding sites. In the absence of ligand, the IRF-hER fusion gene has a low activity (left).

Figure 6 shows an IRF-1-GFP hER expression con-

struct. The eGFP (Promega) reading frame has been inserted in frame into a BamHI restriction site between the reading frames for IRF-1 and hER. IRF-1-GFP hER is expressed from a constitutive viral promoter (P; Dirks et al., 1994).

Figure 7 shows the estrogen regulated transcriptional activation of IRF binding sites containing promoters by IRF-GFP-hER expressing cells. NIH3T3 cells were transfected with two different plasmids expressing IRF-GFP-hER that differ by a small deletion (d) outside of the reading frame, and for comparison IRF-hER. Transient luciferase induction from an ISRE promoter element (pGL2-ISRE-luc) was determined in the presence of 1mM estradiol relative to the luciferase activity in the absence of estradiol of Renilla luciferase from a constitutive viral promoter (CMV-rluc).

Figure 8 shows that IRF-1-GFP-hER fluorescence can be used as to isolate fusion gene expressing single cells by FACS sorting. GFP FACS analysis and sorting of BHK-21 cells (A) and BHK-21 cells transfected with IRF-1-GFP hER (B).

Figure 9 shows the estradiol regulated IRF-1-GFP hER activated gene expression from an IRFE promoter. A stably transfected IRF-1-GFP hER expressing L88-5 single cell clone (L21) isolated by FACS sorting of highly fluorescent cells was transiently transfected using Lipofectin (GIBCO/BRL) with a

firefly luciferase gene expressed for 16 hours from an IRF1 inducible promoter in the absence (L21) or presence of 1 µM estradiol (L21+E2), relative to constitutively expressed renilla luciferase.

Examples for specific applications in industrial relevant systems.

Figures 10 and 11 relate to two examples of a relevant pharmaceutical protein expressing BHK (baby hamster kidney) cell clones with IRF-hER enhanced productivity (pg/cell/day, E2) versus control(pg/cell/day, ctrl) and reduced proliferation (living cells x 10e5/ml, E2) versus controll (living cells, ctrl)

The following is one example for increased IgG antibody productivity in a long-term experiment in IRF-hER proliferation controlled BHK-21 cells in a perfused fermenter with microcarriers showing also high viability. For additional characterization of lesser importance glucose consumption and lactate production was determined.

Name: Fermenter 3

Conditions: IgG 8 cells, Cytodex 3 microcarrier, 6g/l, medium for all fermenters: DMEM: F12 with 5% serum and 500 µg/ml G418 and 3 µg/ml Puro (with added dextran and Pluronic)

Fermenter: New Brunswick, stirred tank, 5 l tank capacity, 3.5 l working volume, settling tube as separator (a glas tube in which the microcarriers settle down by gravitational action) while the medium is removed. Works effective up to a perfusionrate of 1 volume/day (also 3.5 l/day). For higher values gravitational action is not sufficient to compensate sucking action.

At the end of the induction stirring was stopped for 20 min. After the microcarriers have settled down the medium was removed by suction except for 0.5 l, added up to 2 l (=wash),

again removed by suction except for 0.5 l and added up to 3.5 l. Approximatly 7% of the initially content of E2 remains (since, however, during induction perfusion was activated, the actual value for E2 was below 7%). The washing procedure tooks about 1-1.5 h.

In all fermenters the perfusion rate was in the range from 0 to 0.9, wherein most of the time the rate was in the range 0.6 to 0.9.

Figure 12 shows the results.

Figure 13 shows an example of high glycoprotein quality in IRF proliferation controlled cells.

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Claims

1. Promoter-transactivator system for inducible high-level mammalian gene expression with the option of cell growth control comprising

(a) a promoter construct (IRFE promoter) having the general structure:

→mRNA

[MPSV-E]-[IRF-1-binding sites]-[CMV]—DNA, wherein

MPSV-E means MPSV enhancer repeats of the sequence:
GCTAGCTTAAGTAACGCCATTTTGCAAGGCATGGGAAAAATACATAACTGAGAATAGAGAAG
TTCAGATCAAGGTCAGGAACAGAGAAACAGGAGAATATGGGCCAAACAGGATATCTGTGGTA
AGCAGTTCCTGCCCCGCTCAGGGCCAAGAACAGTTGGAACAGGAGAATTGGGCCAAACAGGA
TATCTGTGGTAAGCAGTTCCTGCCCCGCTCAGGGCCAAGAACAGATGGTCCCCAGATGCGGT
CCCGCCCTCAGCAGTTTCTAGA,

or isofunctional variants thereof obtained by substitution, insertion or deletion of one or more nucleotides,

IRF-1-binding sites means the sequence:
GATCCCTTCTCGGGAAATGGAAACTGAAAATCAGATCCCTTCTCGGGAAATGGAAACTGAAA
ATCAGATC,

or isofunctional variants thereof obtained by substitution, insertion or deletion of one or more nucleotides, and

CMV means a minimal promoter of the sequence:
TGGCGTGTACGGTGGGAGGCCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAAACCGTCAA
ACCGCGGAAGCT,

or isofunctional variants therof obtained by substitution, insertion or deletion of one or more nucleotides,

and

(b) a transactivator construct coding for a fusion protein comprising IRF-1 and the estrogen receptor.

2. Expression vector(s) comprising a promoter construct and/or a transactivator construct according to claim 1.

3. Mammalian cells transfected or transformed with an expression vector(s) according to claim 2.

4. Process for inducible high-level mammalian gene expression with the option of cell growth control comprising the steps of

(a) transfecting or transforming mammalian cells with an expression vector or expression vectors, respectively, according to claim 2,

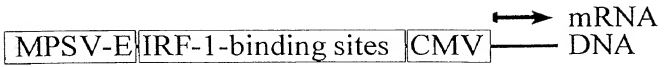
(b) culturing said mammalian cells, or transfected or transformed mammalian cells according to claim 3, in a suitable medium, and,

(c) optionally, controlling the growth of said mammalian cells by varying the concentration and the duration of exposure to estradiol in the medium.

Summary

A promoter-transactivator system achieves regulated high-level gene expression in proliferation-controlled mammalian cells. The novel composite promoter contains constitutive enhancer elements that allows a basal expression level as high as the levels achieved with a very efficient viral promoter. In addition, the promoter encodes sequences that are bound by a transactivator whose activity can be regulated. By the simple addition of medium supplements expression levels can be achieved above those from the conventional promoter.

Figure 1



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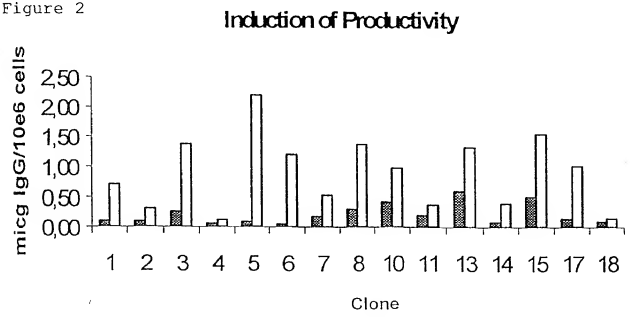

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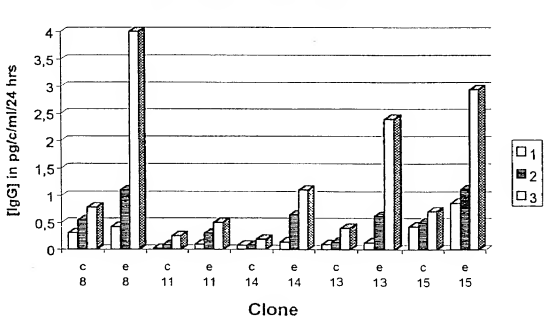

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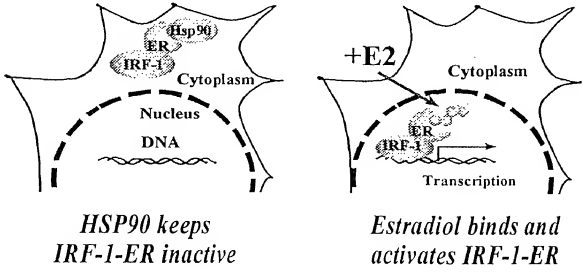

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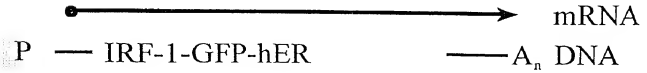
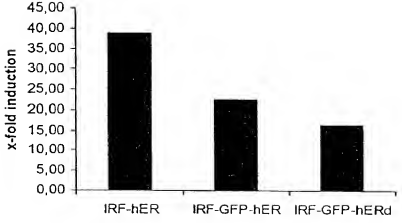


Figure 6

Figure 7



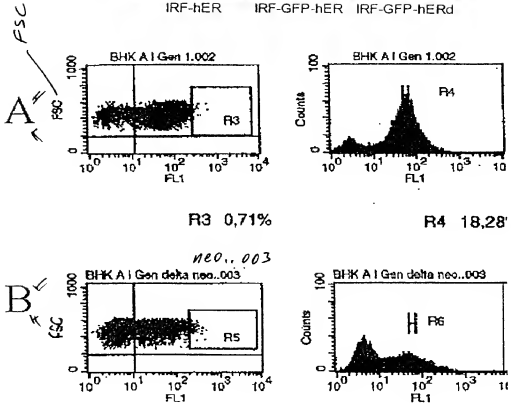
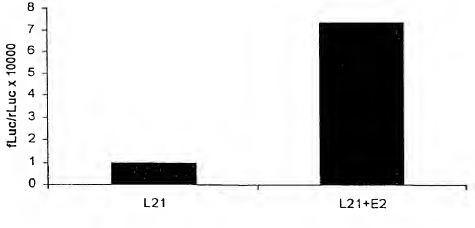
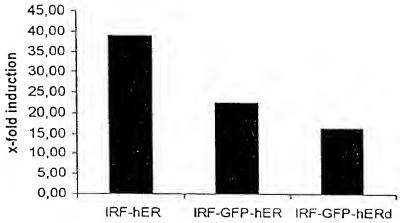


Figure 8

Figure 9





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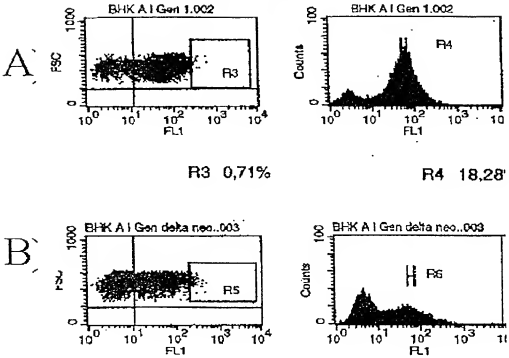

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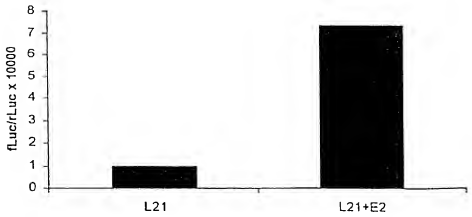


Figure 9

Figure 10 4/7

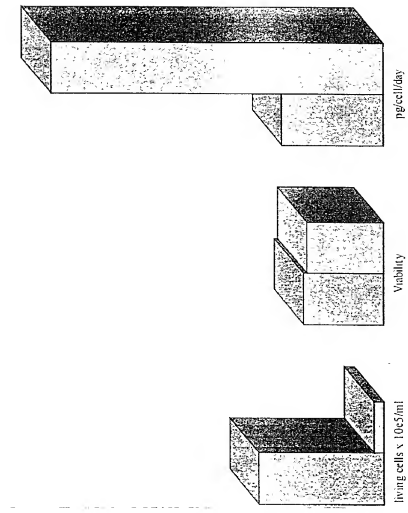

FVII producer clone Nr. 12
cells x 10e5/ml; fraction viable cells; specific productivity in pg/cell/day
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
living cells x 10e5/ml
Viability
pg/cell/day
Ctrl
F2

Figure 11 5/7

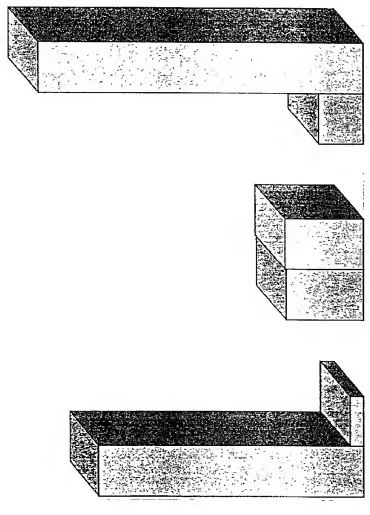
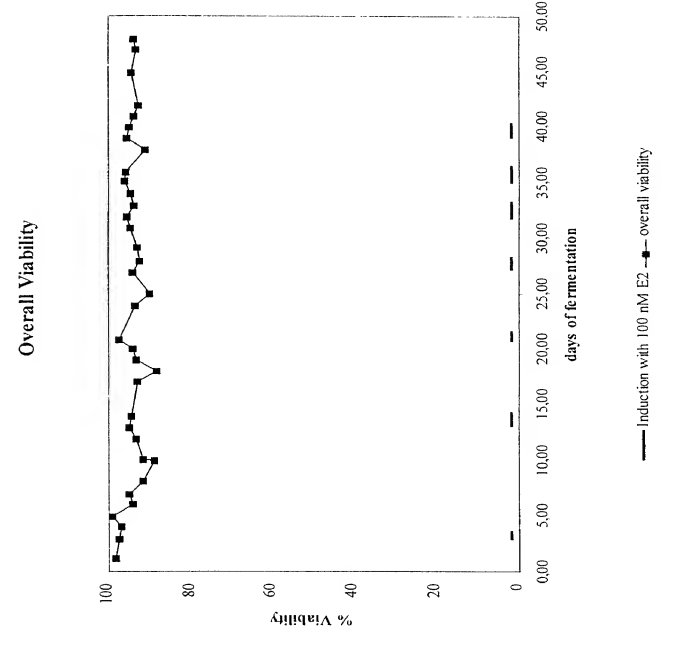


Fig. 12


Overall Viability
% Viability
100
80
60
40
20
0
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
days of fermentation
Induction with 100 nM E2
overall viability

Product Consistency in Proliferation Controled Cells
EPO Std U
0-4hrs -E U
0-4hrs +E
4-8hrs -E
4-8hrs+E
8-24hrs -E
8-24hrs +E
24-32hrs -E
24-32hrs +-E
32-48hrs +-E
Untreated
PNGase
TFA
The glycosyation remains constant

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Under 37 CFR § 1.63; includes reference to PCT International Applications)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED:

Promoter-Transactivator System for Inducible High-Level Mammalian Gene Expression With the Option of Cell Growth Control

the specification of which:

- ☐ is attached hereto
- ☐ was filed on _____ as:
- ☐ United States Application Serial No.
- ☒ as a National Phase or Continuation or Continuation-in-Part or Divisional of PCT Application No. PCT/EP00/03638 (USSN _____), filed April 20, 2000 and designating the U.S., and published as WO 00/65074 on November 2, 2000
- ☐ with amendments through _____ (if applicable, give details).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Country (or PCT)	Application Number:	Filed (Day/Month/Year)	Priority Claimed: Yes	No
Europe	EP 99 108 068.0	23/04/1999	☒	☐
			☐	☐

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States application listed below:

_____ (Application Number) (Filing Date)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to

patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.:	Filed (Day/Month/Year)	PCT Application No.	Status (patented, pending, abandoned)

I hereby appoint Ronald R. Santucci, Registration No. 28,988, and Frommer Lawrence & Haug LLP, or their duly appointed associate, my attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and to insert the Serial Number of the application in the space provided above, and specify that all communications about the application are to be directed to the following correspondence address:

Ronald R. Santucci, Esq.
c/o FROMMER LAWRENCE & HAUG LLP
745 Fifth Avenue
New York, NY 10151

Direct all telephone calls to: (212) 588-0800
to the attention of: Ronald R. Santucci

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

INVENTOR(S):

Signature: _____ Date: _____
Full name of sole or first inventor: Peter Mueller
Address: Mascheroder Weg 1,
D-38124 Braunschweig, Germany
Citizenship: Germany

Signature: _____ Date: _____
Full name of 2nd joint inventor (if any): Christoph Geserick
Address: Mascheroder Weg 1,
D-38124 Braunschweig, Germany
Citizenship: Germany

Signature: _____ Date: _____
Full name of 3rd joint inventor (if any): Katharina Schroeder
Address: Mascheroder Weg 1,
D-38124 Braunschweig, Germany
Citizenship: Germany

Signature: _____ Date: _____

Full name of 4th joint inventor (if any): Hansjoerg Hauser
Address: Mascheroder Weg 1,
D-38124 Braunschweig, Germany
Citizenship: Germany

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Under 37 CFR § 1.63; includes reference to PCT International Applications)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED:

Promoter-Transactivator System for Inducible High-Level Mammalian Gene Expression With the Option of Cell Growth Control

the specification of which:

- ☐ is attached hereto
- ☐ was filed on _____ as:
- ☐ United States Application Serial No.
- ☒ as a National Phase or Continuation or Continuation-in-Part or Divisional of PCT Application No. PCT/EP00/03638 (USSN _____), filed April 20, 2000 and designating the U.S., and published as WO 00/65074 on November 2, 2000
- ☐ with amendments through _____ (if applicable, give details).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Country (or PCT)	Application Number:	Filed (Day/Month/Year)	Priority Claimed: Yes	No
Europe	EP 99 108 068.0	23/04/1999	☒	☐
			☐	☐

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States application listed below:

_____ (Application Number) (Filing Date)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to

Express Mail No.EV073688400US 00033333

patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.:	Filed (Day/Month/Year)	PCT Application No.	Status (patented, pending, abandoned)

I hereby appoint Ronald R. Santucci, Registration No. 28,988, and Frommer Lawrence & Haug LLP, or their duly appointed associate, my attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and to insert the Serial Number of the application in the space provided above, and specify that all communications about the application are to be directed to the following correspondence address:

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INVENTOR(S):

Signature: _____ Date: 1/24/02

1-80 Full name of sole or first inventor: Peter Mueller
Address: Mascheroder Weg 1,
D-38124 Braunschweig, Germany DEX
Citizenship: Germany

Signature: _____ Date: 7 2.02

2-80 Full name of 2nd joint inventor (if any): Christoph Geserick
Address: Mascheroder Weg 1,
D-38124 Braunschweig, Germany DEX
Citizenship: Germany

Signature: _____ Date: 30. Jan 2002

3-80 Full name of 3rd joint inventor (if any): Katharina Schroeder
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D-38124 Braunschweig, Germany DEX
Citizenship: Germany

Signature: Hansjoerg Hauser Date: 23 Jan. 2002

4-w

Full name of 4th joint inventor (if any): Hansjoerg Hauser
Address: Mascheroder Weg 1,
D-38124 Braunschweig, Germany DEX
Citizenship: Germany